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Sequential injection spectrophotometric system for evaluation of mushroom tyrosinase-inhibitory activity

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ABSTRACT

A sequential injection (SI) spectrophotometric method with absorbance detection at 475 nm has been developed for evaluating activity of some compounds on an inhibition of the mushroom tyrosinase. The method involved a reaction of 3,4-dihydroxyphenylalanine (L-DOPA) and mushroom tyrosinase to form the *o*-dopaquinone. The decrease of the *o*-dopaquinone was related to an increase of tyrosinase-inhibitory activity. Under the optimum conditions (concentration and volume of L-DOPA and mushroom tyrosinase of 2.0 mM, 60 μL and 142 U mL^{-1}, 15 μL, respectively), some antioxidant compounds were examined for the tyrosinase-inhibitory activity. A batch enzymatic assay of tyrosinase-inhibitory activity was applied as the reference method for comparison. The results of IC_{50} values obtained from the proposed method and the batch method were correlated well, with r^2 of 0.969. The SIA provides higher precision and degrees of automation, consumes smaller amounts of chemicals and it is simpler and faster than the batch method.

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1. Introduction

Tyrosinase (EC 1.14.18.1) is a copper-containing enzyme which is a key enzyme in melanin biosynthesis. This enzyme has three domains, and the central domain contains two copper binding sites which are the active site in the tyrosinase catalytic reaction. Tyrosinase catalyzes two distinct reactions: the hydroxylation of tyrosine by monophenolase action (cresolase or monophenolase activity) and the oxidation of 3,4-dihydroxyphenylalanine (L-DOPA) to *o*-dopaquinone by diphenolase action (catecholase or diphenolase activity) [1]. Melanin is one of the most widely distributed pigments and is found in bacteria, fungi, plants and animals. It is a heterogeneous polyphenol-like biopolymer with a complex structure and color varying from yellow to black. The color of mammalians skin and hair is determined by a number of factors, the most important of which is the degree and distribution of melanin pigmentation [2]. Melanin plays an important role in protecting human skin from a harmful effect of UV radiation from the sun. However, an accumulation of an abnormal amount of melanin in different specific parts of skin resulting in more pigmented patches might become an esthetic problem.

In the food industry, tyrosinase is a very important enzyme in controlling the quality and economics of fruits and vegetables. The enzyme catalyzes oxidation of phenolic compounds to the corresponding quinones and is responsible for an enzymatic browning of fruits and vegetables. These problems generally result in a loss of nutritional and market value. Thus, in cosmetic, medicinal, and food products, tyrosinase inhibitors have become increasingly important to prevent hyperpigmentation and enzymatic browning.

A large number of tyrosinase inhibitors from both natural and synthetic sources have been identified [3,4]. Many tyrosinase inhibitors are examined in the presence of tyrosine or dopa as the enzyme substrate, and activity is assessed in terms of dopachrome formation [5,6]. Thus, experimental observation of tyrosinase inhibitory activity could be accomplished by one of following [7]: (a) reducing agents causing chemical reduction of dopaquinone; (b) *o*-dopaquinone scavenger; (c) alternative enzyme substrates; (d) nonspecific enzyme inactivators; (e) specific tyrosinase inactivators; and (f) specific tyrosinase inhibitors. Among the six types of compounds described above, only the (e) and (f) are regarded as "true inhibitors," which actually bind to the enzyme and inhibit its activity. Several chemicals from plant origin have been tested as cosmetics and pharmaceuticals to

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prevent overproduction of melanin in epidermal layers or as whitening agents. For example: Baurin et al. [8] reported a preliminary screening of some tropical plants for anti-tyrosinase activity, this study indicated that the almost extracts present a poor anti-tyrosinase activity (less than 50%). About 18% of plant extracts present strong inhibition of tyrosinase. Many flavonols have been isolated from plants, and some of them were identified as tyrosinase inhibitors. Quercetin and its derivatives have been found to inhibit the oxidation of L-DOPA catalyzed by the mushroom tyrosinase that is shown the inhibition of melanin formation [9–11]. Kojic acid, 5-hydroxy-2-(hydroxymethyl)-4-pyrone, is a natural product found in fermented food; it has been widely used as a food additive and preservative and as a skin-whitening agent in cosmetics [12,13].

Many techniques were reported for the evaluation of tyrosinase-inhibitory activity [14–16], however the methods involved the use of high cost chemicals, thus it is interesting to develop a method that can decrease the amount of reagents used. Flow based techniques are widely accepted worldwide for various chemical analyses. Flow injection analysis (FIA) technique in which a sample was injected into a continuous flow stream of reagent was introduced since 1975 in Denmark by Ruzicka and Hansen [17], providing advantages such as fast analysis, low chemical consumption and high degrees of automation. Later, a sequential injection analysis (SIA) was proposed by Ruzicka and Marshall in 1990 [18], of which the microliter volumes of sample and reagent(s) were sequentially aspirated into the system. This technique can greatly reduce amount of chemicals per analysis and hence decrease the generation of waste solution. Araujo et al. [19] presented an automatic flow methodology for kinetic studies of the mushroom tyrosinase-mediated oxidative coupling using caffeic acid (phenolic acid) as a substrate. This method is able to reduce consumption of reagent such as ionic liquid and subsequently minimize waste generation.

In this work, the sequential injection system has been developed for determination of tyrosinase-inhibitory activity. The reaction of L-DOPA (as the enzyme substrate) to produce *o*-dopaquinone which could be determined spectrophotometrically at 475 nm was used to monitor activity of mushroom tyrosinase. The decrease of dopaquinone dye related to an increase of tyrosinase-inhibitory activity. Various antioxidant compounds were evaluated for their tyrosinase inhibitory activity using the developed method and the results were compared with the batchwise tyrosinase enzymatic assay.

2. Experimental

2.1. Chemicals

Mushroom tyrosinase (EC 1.14.18.1, 5,073 U mg^{-1} solid, from mushroom) and epigallocatechin gallate were purchased from Sigma Chemical Co. Ltd. (USA). L-Dihydroxy phenylalanine (L-DOPA) was from Aldrich Chemical Co. Ltd. (USA). Quercetin was from Tokyo Chemical Industry Ltd. (Japan). Epigallocatechin and anisaldehyde were from Nacalai Tesque Inc. (Japan). Other chemicals (NaOH, $NaH_2PO_4 \cdot 2H_2O$, Na_2HPO_4, ethanol, kaempferol, epicatechin gallate, kojic acid and ascorbic acid) were purchased from Wako Pure Chemical Industries Ltd. (Japan). A Milli-Q water obtained from the water purification system (Millipore, Tokyo, Japan) was used to prepare solutions.

2.2. Instrument setup

The developed sequential injection (SI) system is schematically depicted in Fig. 1(a). It consisted of a syringe pump, a multi-position valve (MGC JAPAN Co., Japan) and a spectrophotometric detector (UV–vis detector S-3702, SOMA OPTICS, Japan). PTFE tube (i.d. 0.8 mm, o.d. 1.58 mm) was used for making a holding coil and a mixing coil, and assembling the system. An output signal from the spectrophotometer was recorded by a chart recorder (SERVOCORDER SR6211, GRAPHTEC Co., Japan). The SIA system was controlled by a computer via the SI controller software (MGC JAPAN Co., Japan).

2.3. Preparation of solutions

Phosphate buffer solution (PBS) pH 6.8 was prepared from NaH_2PO_4–Na_2HPO_4 at 0.1 M total phosphate by dissolving in water. A stock solution of 2.0 mM L-DOPA was prepared by dissolving 9.86 mg L-DOPA with 25.0 mL water. The original mushroom tyrosinase solution of initial activity of 5,073 U mg^{-1} solid was dissolved with PBS to a concentration of 142 U mL^{-1}.

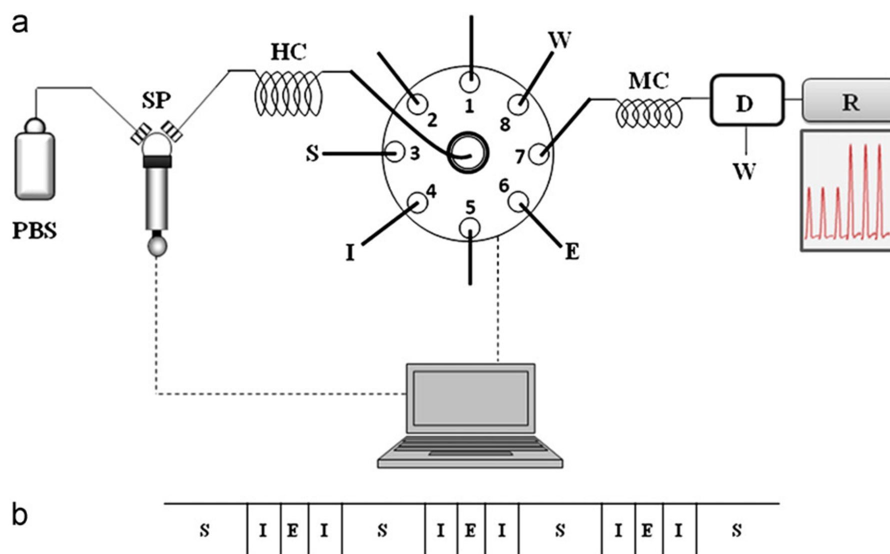

Fig. 1. (a) Manifold of a sequential injection system with spectrophotometric detection for determination of tyrosinase inhibition activity, (b) Sequence of solutions in a holding coil of the SIA system; PBS: phosphate buffer solution, SP: syringe pump, HC: holding coil, E: mushroom tyrosinase, S: L-DOPA substrate, I: inhibitor standard or sample, MC: mixing coil, D: spectrophotometer, W: waste, R: recorder.

Stock solutions of quercetin and kaempferol of 5.0 mM were prepared in 5.0 mL ethanol. Working solution of each chemical was freshly prepared by diluting the stock solution with 50%(v/v) ethanol. Stock solution of epicatechin gallate, epigallocatechin, epigallocatechin gallate of 1.0 mM, kojic acid and anisaldehyde of 5.0 mM were prepared in 5.0 mL of 50%(v/v) ethanol. Working solution of each chemical was then freshly prepared by diluting the respective stock solution with water. A stock solution of ascorbic acid of 5.0 mM was prepared in 5.0 mL water and its working solution was freshly prepared by diluting the stock solution with water. Since different solvents were used to dissolve various compounds leading to different background signals registered at the detector, so that the respective solvent solution was used as a blank solution for background signal correction in the measurement.

2.4. Sequential injection procedure

Employing the SI system as shown in Fig. 1(a), the operational sequence as summarized in Table 1 was used by the software to control the system. In brief, the analytical steps started by filling all the lines with a carrier solution (PBS) and then dispensing the carrier solution to a flow-through cell while recording a baseline signal. Then, L-DOPA, tyrosinase and sample were aspirated to a holding coil with a solution sequence as shown in Fig. 1(b). The zones were mixed and dopaquinone dye was produced. Next, the product solution was dispensed to a flow-through cell to be detected by a spectrophotometer at 475 nm. A decrease in an absorbance of the dopaquinone dye was related to an increase of tyrosinase inhibitor. Peak height of the SIAgram obtained was linearly proportional to the dopaquinone, which was oppositely proportional to the tyrosinase-inhibitory activity.

The tyrosinase-inhibitory activity was expressed as percent inhibition of tyrosinase, calculated as follow:

$$\%\text{Inhibition} = 100 \times (A_0 - A)/A_0$$

where A_0 is a corrected peak height of the assay without an inhibitor and A is a corrected peak height of the assay with an inhibitor (in the sample zone). So, the %inhibition increased when the sample contained high concentration of tyrosinase inhibitor.

The IC_{50} (50% inhibiting concentration) value of the tyrosinase inhibitor was determined by plotting %inhibition (y) versus an inhibitor concentration (x) at the %inhibition close to 50%, *i.e.*, 2 points at lower and higher than 50%inhibition, respectively, yielding a linear equation as follow:

$$y = mx + n$$

where m and n are the slope and intercept of this linear correlation, respectively. IC_{50} can then be calculated for 50%inhibition ($y=50$) such that

$$y = 50 = m(IC_{50}) + n \text{ or } IC_{50} = (50 - n)/m$$

2.5. Enzymatic assay of tyrosinase inhibition

The L-DOPA was used as a substrate in this experiment. The activity of mushroom tyrosinase was determined spectrophotometrically at 475 nm (measurement of *o*-dopaquinone product). First, 1 mL of 2.5 mM L-DOPA solution was mixed with 1.8 mL of 0.1 M phosphate buffer (pH 6.8) and incubated at 25 °C for 10 min. Then, 0.1 mL of the sample solution and 0.1 mL of the tyrosinase solution (142 U mL^{-1}) were consecutively added to the mixture, and the initial rate of the reaction was immediately measured as a linear increase in the absorbance at 475 nm. The change in absorbance was recorded for 3 min at room temperature. The tyrosinase activity was expressed as percent inhibition of tyrosinase, calculated as follow:

$$\%\text{inhibition} = 1 - (B/A) * 100$$

where A is the change in absorbance of the assay without an inhibitor and B is the change in absorbance of the assay with an inhibitor.

3. Results and discussion

An enzymatic assay of tyrosinase (batch method) was preliminarily used to measure tyrosinase activity. This method is based on the oxidation of L-DOPA to *o*-dopaquinone by the mushroom tyrosinase. An increasing of signal was observed at the wavelength of 475 nm, the involved reactions are summarized in Fig. 2. Therefore, in the present work, the analytical wavelength of 475 nm was selected to be used in the SI system. The SI-spectrophotometric system as presented in Fig. 1(a) was used. A common flavonol, quercetin, was used as a standard

Table 1
Protocol sequence for the study of tyrosinase-inhibitory activity.

Step	Valve position	Operation time (s)	Flow rate (μL s^{-1})	Volume (μL)	Description
1	–	5.0	400	2000	Filling syringe with a carrier solution
2	7	20.0	100	2000	Dispensing a carrier solution to detector for recording baseline signal
3	3	1.5	10	15 (×4)	Aspiration of the L-DOPA to HC
4	4	0.3	10	3 (×6)	Aspiration of the sample to HC
5	2	0.5	10	5 (×3)	Aspiration of the tyrosinase to HC
				Sum=93	(Steps 3–5 were repeated according to sequence in Fig. 1(b))
6	–	30	100	3000	Filling syringe with a carrier solution
7	7	31	100	3093	Dispense HC content towards the detector

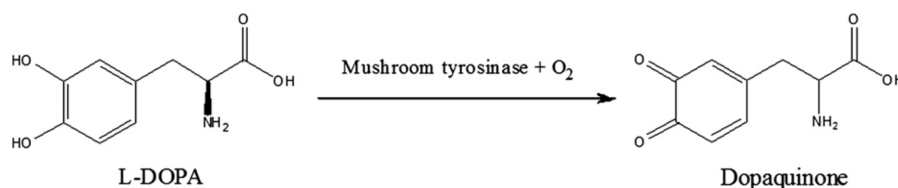

Fig. 2. Oxidation of L-DOPA to *o*-dopaquinone by mushroom tyrosinase.

inhibitor in both the batch and SI systems; it could inhibit the tyrosinase, thus a decrease in an absorbance in the batch system and a decrease of peak height in the SI system were observed. The signal obtained was inversely proportional to the tyrosinase inhibitory activity, so the decrease of signal was observed when the sample solution contains the tyrosinase inhibitor.

The sequence of solution is an important parameter in SI system because it affects the mixing between various zones of solutions; from a preliminary study a sequence as shown in Fig. 1(b) was selected to use in further experiments. In the selected sequence, the inhibitor standard (or sample) was inserted between substrate and enzyme, thus the enzyme was efficiently mixed with the inhibitor leading to efficient inhibition. Sample volume of 18 μL was selected in order to minimize interference from color and turbidity of the sample. The important parameters were optimized such as the concentrations and volumes of substrate and enzyme and the delay time (reaction time). In each of these experiment, carrier buffer, temperature and detection system were kept constant as follow: a 0.1 M PBS, 25 °C and a spectrophotometer (475 nm), respectively.

3.1. Optimization of concentration and volume of the substrate

The L-DOPA substrate concentration was studied in the range of 0.0–5.0 mM while its volume was fixed at 100 μL. The peak height linearly increased up to 3.0 mM L-DOPA concentration and then level off with further increase of L-DOPA concentration because the enzyme became saturated with the L-DOPA substrate (Fig. 3). However, 1.0, 1.5, 2.0, 2.5 and 3.0 mM of L-DOPA were selected for investigation on the inhibition with 0.8 mM quercetin. The 2.0 and 3.0 mM of L-DOPA concentration showed higher %inhibition, thus these concentrations were selected for optimization of a volume of L-DOPA solution in the range of 60–200 μL, with quercetin of 0.8 mM. In this study, the volume of L-DOPA solution lower than 60 μL was not investigated because it provided low sensitivity (small peak height). The results as shown in Fig. 4 indicated that a 60 μL volume gave the highest %inhibition for both the concentrations of L-DOPA. The 2.0 mM L-DOPA had %inhibition higher than 3.0 mM. As a reason, quercetin is a competitive inhibitor [2] that is the chemical structure similar to the substrate. It competes with the substrate for the same active site on an enzyme molecule. Therefore, the enzyme inhibitory activity is inversely dependent on the amount of the substrate when the concentration of inhibitor and enzyme were fixed. Thus, 60 μL of 2.0 mM L-DOPA was selected for further optimizations.

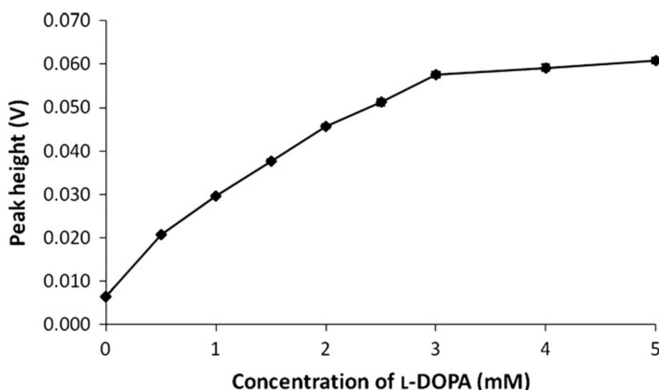

Fig. 3. Effect of L-DOPA concentration on peak height of the SIAgram; volume of L-DOPA solution, 60 μL; tyrosinase, 142 U mL^{-1}/ 60 μL.

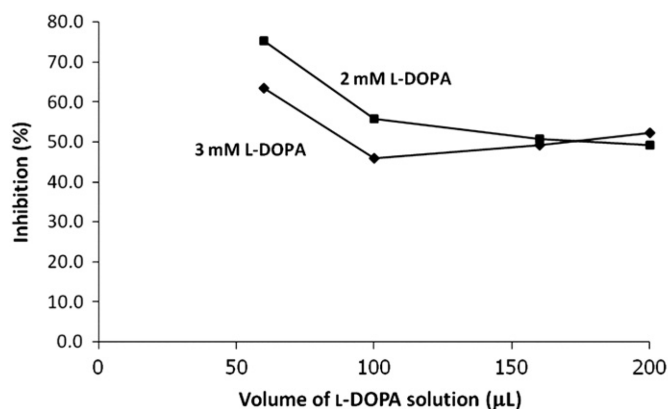

Fig. 4. Effect of the volume of L-DOPA solution on tyrosinase inhibition; tyrosinase, 142 U mL^{-1}/ 60 μL; quercetin, 0.8 mM.

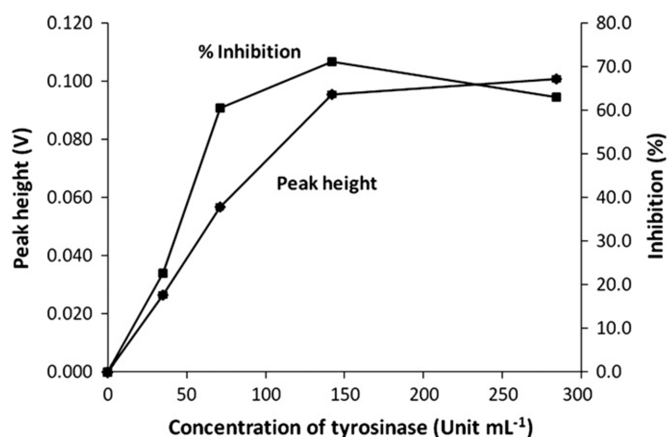

Fig. 5. Effect of tyrosinase concentration on peak height and %inhibition; quercetin, 0.8 mM.

3.2. Optimization of the enzyme activity

Concentration and volume of a mushroom tyrosinase were studied. Firstly, the tyrosinase concentration was varied from 35 to 284 U mL^{-1} while its volume was fixed at 30 μL. It was found that both the peak height and %inhibition increased dramatically with the increase of tyrosinase concentration and started to level off at about 150 U mL^{-1} (Fig. 5). The slight decrease of %inhibition at too high enzyme concentration was caused by the effect of enzyme on decreasing competitive of inhibitor. Quercetin is a competitive inhibitor [2], which closely resembles the chemical structure and molecular geometry of the substrate. The inhibitor competes with the substrate for the same active site of enzyme molecule. The inhibitor may interact with the enzyme at the active site, but no reaction takes place. Therefore, the degree of enzyme inhibition depends upon the enzyme concentration when the concentration of inhibitor and substrate is fixed. So the increasing enzyme concentration, the competition between inhibitor and substrate was decreased. Thus, the concentration of 142 U mL^{-1} tyrosinase was selected. Then, volume of tyrosinase solution was investigated from 15 to 90 μL, with the quercetin in the range of 0.2–0.8 mM. It was found that %inhibition decreased with increasing the volume of tyrosinase solution, and an increase of peak height was observed. Therefore, 15 μL of tyrosinase solution was chosen as it resulted in higher %inhibition with an adequate sensitivity of detection and using a lower amount of an expensive enzyme.

Table 2
The studied ranges and optimum conditions of SI system.

Parameter	Studied range	Selected value
L-DOPA concentration (mM)	0–5.0	2.0
Volume of L-DOPA solution (μL)	60–200	60
Tyrosinase concentration (U mL^{-1})	0–248	142
Volume of tyrosinase solution (μL)	15–90	15
Delay time (s)	0–40	0

3.3. Optimization of the delay time

In the SI system, timing could be controlled precisely. The delay time after solutions were mixed is related to the reaction time. This parameter was studied between 0 and 40 s, it indicated that the peak heights (sensitivity) were slightly increased whereas the %inhibitions were not significantly different. Although the delay time of 40 s provided the highest sensitivity, the sample frequency was the lowest. So, the procedure without the delay time was selected in order to increase sample frequency. The actual reaction time included the time during the solutions being in the HC and transport from HC to the detection flow cell, which was less than 60 s (steps 3–7 in Table 1).

3.4. Precision and sample throughput

The sequence and optimum conditions of the SI system were summarized in Tables 1 and 2, respectively. Precision of the system was studied by 7 replicated injections of 100 μM quercetin, a relative standard deviation (RSD) of 3.50% was obtained. Different concentrations of kojic acid which has higher tyrosinase inhibitory activity was also injecting into the system. RSDs of 1.15%, 0.85% and 1.36% were obtained for 10.0 μM, 5.0 μM and 0.0 μM kojic acid, respectively. The method had a sample throughput of 20 h^{-1}, with consumption of 3.0, 0.060, 0.015 and 0.018 mL of PBS solution, L-DOPA, tyrosinase and sample solutions, respectively, per analysis.

3.5. Investigation of tyrosinase-inhibitory activity of various antioxidant compounds

The inhibitory activity of the enzyme by the inhibitor could be normally reported in 2 ways, i.e., (1) expressed as activity as equivalent to concentration of the standard inhibitor and (2) expressed as concentration of that inhibitor that could give 50%inhibition of the enzyme (IC_{50}). The IC_{50} value should be more appropriate because the inhibition by different compounds may involve different mechanisms, so it should not be easily compared with the standard inhibitor. IC_{50} is also more valuable for comparison of activity and the utilization of different compounds. The determination of IC_{50} value should be done on purified compounds. However, the IC_{50} value obtained may vary a lot, depending on experimental conditions. By using SI system, precision and accuracy of the results could be improved.

Under the optimum conditions of the SI-spectrophotometric system, eight common antioxidant compounds including quercetin, kaempferol, epigallocatechin (EGC), epicatechin gallate (ECG), epigallocatechin gallate (EGCG), anisaldehyde, kojic acid and ascorbic acid were selected for investigation of their tyrosinase-inhibitory activities. Some antioxidant compounds having color and turbidity could affect the background signal of the proposed system when presented at high concentration. Therefore, a sufficient dilution of the sample was needed in order to avoid this problem. In addition, solvent for dissoving the compounds also showed a background signal, thus a blank correction had to be carried out. To do this, PBS, L-DOPA and blank solutions were aspirated, mixed and pushed through the detection system to record a background signal. Then, sample analysis was performed by aspirating PBS, L-DOPA, sample and mushroom tyrosinase solutions into the system; *o*-dopaquinone was produced, the tyrosinase-inhibiting signal was observed. This signal was subtracted by the background signal before the %inhibition of tyrosinase by the antioxidant compounds was calculated, as described in section 2.4. Although 50%(v/v) ethanol could reduce enzyme activity to about 50%, it must be used for dissolving of some compounds. By subtracting background signal that caused by solvent, inhibitory activity of the compounds could be estimated.

The inhibition of tyrosinase by these compounds was expressed as the concentration that resulted in half maximal enzyme activity (IC_{50}), the results were presented in Table 3. The kojic acid, ascorbic acid and quercetin had lower IC_{50} values; 10.1, 36.1 and 74.8 μM, respectively, thus these compounds showed the strongest inhibition of mushroom tyrosinase. While the epicatechin gallate and anisaldehyde were less inhibitory activity when compared in this group. According to another research, Kubo et al. [20] also reported the IC_{50} values of kojic acid (IC_{50}=14 μM) and quercetin (IC_{50}=70 μM) that showed the strongest inhibition of tyrosinase. Considering structure of compounds in Fig. 6, the hydroxyl (OH) substitutions in the A and B rings in the flavonoids, are crucial for Cu^{2+}-chelate formation, thus the tyrosinase inhibitory activity should depend on OH group [21]. Comparing between quercetin and kaempferol, the kaempferol exhibited a lower inhibitory activity, due to the lack of 3′–OH groups. In case of EGC, ECG and EGCG, the inhibitory effect of ECG and EGCG were weaker than EGC. A possible reason for the decreased inhibitory activity observed was a steric hindrance effect of the C3-substitution in C-ring, which could hinder the approach of large molecules to the active site of the enzyme. Other compounds, kojic acid contained two OH groups and had a small structure, so it was proved to be a good tyrosinase inhibitor, while the anisaldehyde was absent of –OH groups that gave the lowest inhibition of tyrosinase with IC_{50} value higher than 200 μM. Ascorbic acid is a good reducing agent or contained many OH groups, so that it gave higher %inhibition, with IC_{50} value lower than 40 μM.

An enzymatic assay of tyrosinase inhibition (batch method) was applied as the reference method for comparison of evaluating the tyrosinase-inhibitory activity. Both the SI method (y) and the batch method (x) were correlated well as indicated by a linear equation: $y=0.8185x+18.69$; $r^2=0.969$. The experimental t-value between both of the methods was 1.10 which was less than the critical t-value of 2.45 for 6° of freedom at the 95% confidence level. Although the same chemistry has been used in SI and batch systems, the conditions are not identical, such as the ratio of substrate [S] and inhibitor [I] is different. In case of quercetin, a competitive inhibitor, which compete with substrate for binding

Table 3
The IC_{50} values on tyrosinase-inhibitory activity of antioxidant compounds using the SI method compared with the batch method.

Antioxidant compound	IC_{50} value (μM)	
	SI	Batch
Quercetin	74.8 ± 5.1	43.4 ± 1.3
Kaempferol	177.1 ± 2.5	176.9 ± 6.1
Epicatechin gallate	233.5 ± 12.5	289.6 ± 27.6
Epigallocatechin	85.7 ± 1.6	80.3 ± 2.0
Epigallocatechin gallate	105.5 ± 0.8	97.2 ± 0.2
Anisaldehyde	258.1 ± 8.1	279.3 ± 9.0
Kojic acid	10.1 ± 0.1	14.2 ± 0.3
Ascorbic acid	36.1 ± 1.7	34.7 ± 1.4

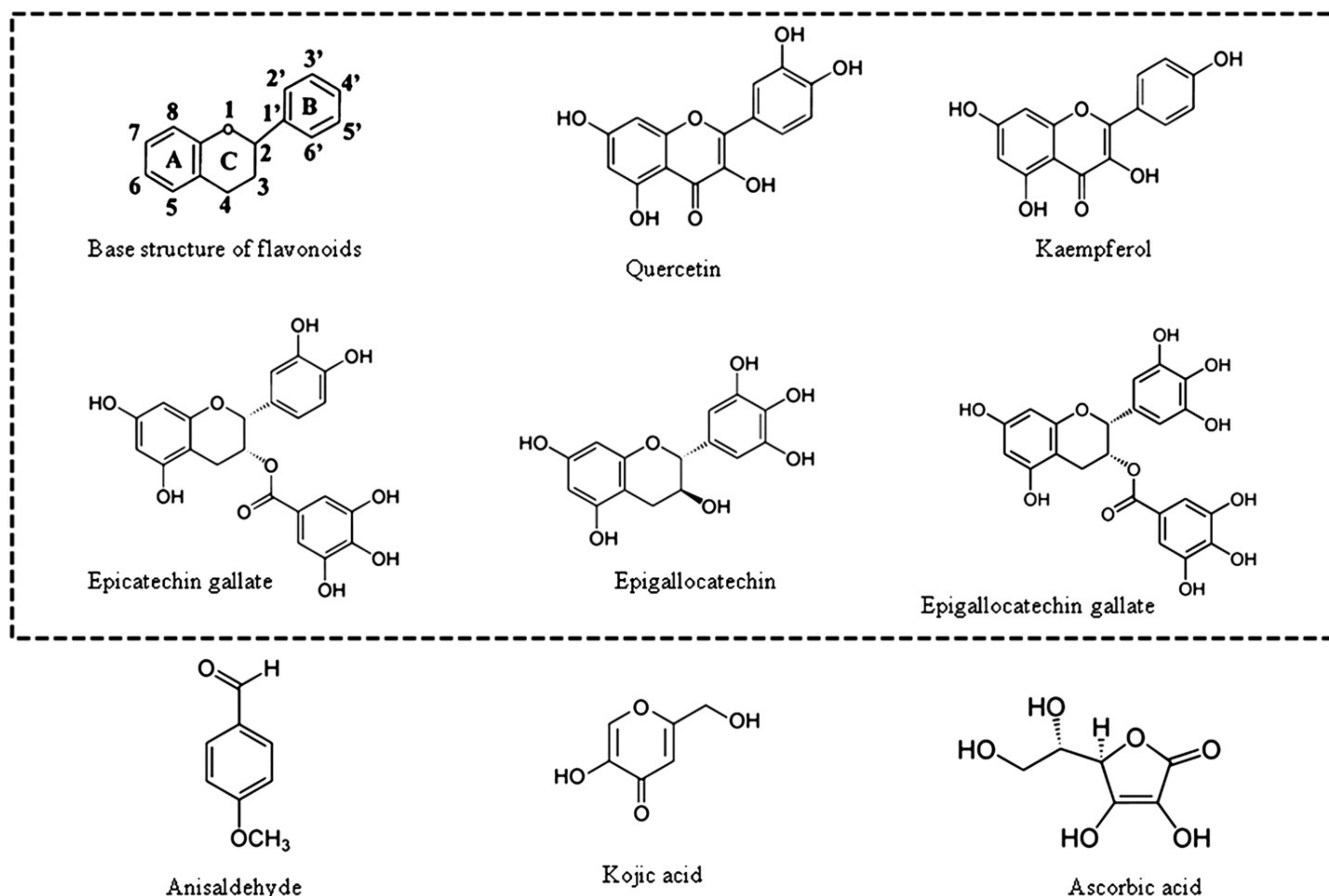

Fig. 6. Structure of some tyrosinase inhibiting compounds.

Table 4
Comparison of batch-wise method and the developed method for evaluation of tyrosinase- inhibitory activity of various compounds.

System	Substrate	Tyrosinase concentration (U mL^{-1})	Volume of tyrosinase solution (μL)	Tyrosinase amount (U)	Inhibitor/ sample	Ref.
Spec. (475 nm)	L-DOPA, L-Tyrosine	1,600	50	80	Some tropical plants	[8]
Spec. (492 nm)	L-Tyrosine	1,000	7	7	Green tea	[22]
Spec. (475 nm)	L-DOPA	138	100	10.1	Citrus essential oils	[1]
Spec. (475 nm)	L-DOPA	138	100	10.1	Some flavonols	[20]
Spec. (475 nm)	L-DOPA	138	100	10.1	Anise oil	[23]
Spec. (475 nm)	L-DOPA	138	100	10.1	Olive oil flavor compounds	[24]
Spec. (475 nm)	L-DOPA	138	100	10.1	Anisaldehyde	[25]
Spec. (475 nm)	L-DOPA	314.8	50	15.7	Fresh tea leaves	[26]
Spec. (475 nm)	L-DOPA, L-Tyrosine	333	30	10	Licorice roots	[27]
SI-Spec. (475 nm)	L-DOPA	142	15	2.1	Some antioxidant compounds	This work

L-DOPA, 3,4-dihydroxyphenylalanine; SI, sequential injection; Spec., spectrophotometry.

to tyrosinase, the higher ratio of $[S]/[I]$ in SI than in batch method may lead to higher IC_{50} value in SI method. The procedure for estimation of IC_{50} value was also cause variation in IC_{50} value obtained. Even in batch method, high variation IC_{50} values have been reported, such as 26.5–130 μM for quercetin, 7.75–70 μM for Kojic acid, and 170–230 μM for Kaempferol. In the developed SI method, IC_{50} were evaluated by using 2-points close to 50%inhibition (at lower and higher than 50%inhibition, respectively), leading to obtain more accurate value. At 50%inhibition of some inhibitors, there are not in the linear relationship ranges of %inhibition versus inhibitor concentration, hence the use of 2 points far from 50%inhibition should give inaccurate IC_{50} value. In batch method only two points which may be far from 50%inhibition are normally used for estimation of IC_{50}, because of the high fluctuation of the absorbance signal measured in batch method may cause high variation of IC_{50} value when the points close to 50%inhibition were used. The tedious operation of batchwise assay also cause difficult to find out inhibitor concentration that give more accurate IC_{50} value.

Despite a mushroom tyrosinase has been widely used due to advantages of ease of purchase, purity, and low price compared with a human tyrosinase, the reactions of various substrates and

inhibitors to the mushroom tyrosinase might be different from the human one. Therefore, the further studies on human tyrosinase should be interested if the consumption of the enzyme could be reduced. Comparison of the tyrosinase consumption in batch-wise methods with the developed method for evaluating tyrosinase-inhibitory activity of various compounds was presented in Table 4. It is obviously showed that the developed SI method is able to reduce tyrosinase consumption, which is saving cost of analysis. Therefore, it is a promising tool for the study on the human tyrosinase-inhibitory activity.

4. Conclusions

In this work, we developed the SI system using L-DOPA-mushroom tyrosinase reaction for evaluation of tyrosinase-inhibitory activity of various antioxidant compounds. The system consumes only small amount of chemicals, especially the enzyme which is expensive and has high degrees of automation, allowing precise measurement of the time dependent absorbance which is tediously to be done in a batch system. The experimental results indicated that the inhibitory activity expressed as IC_{50} values obtained from the proposed and batch methods were in good correlation. In addition, the developed method was more convenient, high sensitivity and precision, consumed smaller amount of chemicals and produced lower amount of waste. The SI system could be usefully applied to agricultural and natural products.

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References

[1] R. Matsuura, H. Ukeda, M. Sawamura, J. Agric. Food Chem. 54 (2006) 2309.
[2] Y.J. Kima, H. Uyamab, Cell. Mol. Life Sci. 62 (2005) 1707.
[3] O. Nerya, R.B. Arie, T. Luzzatto, R. Musa, S. Khativ, J. Vaya, Postharvest Biol. Technol. 39 (2006) 272–277.
[4] F. Zocca, G. Lomolino, A. Lante, Food Res. Int. 44 (2011) 957–963.
[5] P. Han, C. Chen, C. Zhang, K. Song, H. Zhou, Q. Chen, Food Chem. 107 (2008) 797–803.
[6] L. Xie, Q. Chen, H. Huang, X. Liu, H. Chen, R. Zhang, Int. J. Biochem. Cell 35 (2003) 1658–1666.
[7] T. Chang, Int. J. Mol. Sci. 10 (2009) 2440–2444.
[8] N. Baurin, E. Arnoult, T. Scior, Q.T. Do, P. Bernard, J. Ethnopharmacol. 82 (2002) 155–158.
[9] I. Kubo, T. Nitoda, K. Nihei, Molecules 12 (2007) 1045–1056.
[10] E. Tangke Arung, S. Furuta, H. Ishikawa, I.W. Kusuma, K. Shimizu, R. Kondo, Food Chem. 124 (2011) 1024–1028.
[11] Q. Chen, I. Kubo, J. Agric. Food Chem. 50 (2002) 4108–4112.
[12] X. Yang, H. Zhang, Food Chem. 102 (2007) 1223–1227.
[13] G.A. Burdock, M.G. Soni, I.G. Carabin, Regul. Toxicol. Pharmacol. 33 (2001) 80–101.
[14] A.M. Girelli, E. Mattei, A. Messina, Biomed. Chromatogr. 18 (2004) 436–442.
[15] S. Wangthong, I. Tonsiripakdee, T. Monhaphol, R. Nonthabenjawan, S.P. Wanichwecharungruang, Biomed. Chromatogr. 21 (2007) 97–100.
[16] Z. Yang, Y. Zhang, L. Sun, Y. Wang, X. Gao, Y. Cheng, Anal. Chim. Acta 719 (2012) 87–95.
[17] J. Ruzicka, E.H. Hansen, Anal. Chim. Acta 78 (1975) 145–157.
[18] J. Ruzicka, G.D. Marshall, Anal. Chim. Acta 237 (1990) 329–343.
[19] A.R.T.S. Araujo, M.L.M.F.S. Saraiva, J.L.F.C. Lima, Anal. Chim. Acta 690 (2011) 101–107.
[20] I. Kubo, I.K. Hori, S.K. Chaudhuri, Y. Kubo, Y. Sanchezb, T. Ogurab, Bioorg. Med. Chem. 8 (2000) 1749–1755.
[21] D. Kim, J. Park, J. Kim, C. Han, J. Yoon, N. Kim, J. Seo, C. Lee, J. Agric. Food Chem. 54 (2006) 937.
[22] J.K. No, D.Y. Soung, Y.J. Kim, K.H. Shim, Y.S. Jun, S.H. Rhee, T. Yokozawa, H.Y. Chung, Life Sci. 65 (1999) 242.
[23] I. Kubo, I.K. Hori, J. Agric. Food Chem. 46 (1998) 1268.
[24] I. Kubo, I.K. Hori, J. Agric. Food Chem. 47 (1999) 4575.
[25] T.J. Ha, S. Tamura, I. Kubo, J. Agric. Food Chem. 53 (2005) 7024.
[26] S. Sangsrichan, R. Ting, Sci. J. UBU 1 (2010) 78.
[27] O. Nerya, J. Vaya, R. Musa, S. Izrael, R.B. Arie, S. Tamir, J. Agric. Food Chem. 51 (2003) 1202–1203.